

Amendments to the Specification:

Please replace the paragraph beginning at page 4, line 27 as with the following amended paragraph:

In the present invention, the ITS sequence is employed as a marker to distinguish strains and correspondingly classify the pathogenicity of isolates of Rhizoctonia. The ITS information enables phylogenetic location of protective isolates, and design of specific primers for the identification of new isolates. In particular, preferred strains of this invention incorporate include those obtained by PCR using primers directed to at least one of the following target sequences SEQ ID NO: 1 to SEQ ID NO: 14.

Please replace the paragraph beginning at page 14, line 15 as with the following amended paragraph:

For future work, from the sequences of Figure 1, it is possible to ~~pick-out~~ obtain sequences suited for use as PCR primers, as follows directed to at least one of the following target sequences:

Amendments to the Claims:

This listing of claims replaces all prior versions and listings of claims in the application:

Listing of Claims:

Claims 1 through 10 are cancelled.

11. (New) A method for the protection of a plant from pathogenic fungi, the method comprising, providing a binucleate *Rhizoctonia* strain, characterized as having an ITS1 sequence which is at least 90% homologous with SEQ ID NO:20; and allowing the binucleate *Rhizoctonia* strain to grow with the plant.
12. (New) The method of claim 11 where the plant is selected from the group consisting of tomato, pepper, cucumber, lettuce, radish, bean, potato, sugar beet, carrot, garlic, onions, alfalfa, grass, wheat and rape.
13. (New) The method of claim 11, where the plant is an herb.
14. (New) The method of claim 13, where the plant is parsley.
15. (New) The method of claim 11, where the plant is a tree.
16. (New) The method of claim 15, where the plant is a pine tree.
17. (New) The method of claim 11, where the plant is a flower.

18. (New) The method of claim 11, wherein the plant is a field crop, container-grown crop, water bed crop, vegetable crop, fruit crop, ornamental crop, nursery crop, garden crop, forest crop, or greenhouse crop.

19. (New) The method of claim 11, comprising inoculating plant seeds with the binucleate *Rhizoctonia* strain.

20. (New) The method of claim 19, further comprising allowing the seeds to be colonised by the binucleate *Rhizoctonia* strain.

21. (New) The method of claim 19, further comprising allowing the seeds to be colonised by the binucleate *Rhizoctonia* strain prior to planting the seeds.

22. (New) The method of claim 19, further comprising allowing the seeds to be colonised by the binucleate *Rhizoctonia* strain prior to exposure of the seeds to pathogenic fungi.

23. (New) The method of claim 19, further comprising allowing the seeds to be colonised by the binucleate *Rhizoctonia* strain prior to germination.

24. (New) The method of claim 11, wherein providing includes selling the binucleate *Rhizoctonia* strain to a farmer.

25. (New) The method of claim 11, wherein providing includes selling the binucleate *Rhizoctonia* strain to an entity other than a commercial grower.

26. (New) A method of selecting a sample of a binucleate *Rhizoctonia* strain having the ability to protect a plant from pathogenic fungi, the method comprising:

providing a candidate sample of the binucleate *Rhizoctonia* strain to be evaluated for the ability to protect a plant; and

determining if the binucleate *Rhizoctonia* candidate strain has an ITS1 sequence which is at least 90% homologous with SEQ ID NO:20,

if the candidate strain has an ITS1 sequence which is at least 90% homologous with SEQ ID NO:2 then selecting it,
thereby selecting a binucleate *Rhizoctonia* strain having the ability to protect a plant from pathogenic fungi.

27. (New) The method of claim 26, wherein the plant is selected from the group consisting of tomato, pepper, cucumber, lettuce, radish, bean, potato, sugar beet, carrot, garlic, onions, alfalfa, grass, wheat and rape.

28. (New) The method of claim 26, wherein the plant is an herb.

29. (New) The method of claim 26, wherein the plant is a tree.

30. (New) The method of claim 26, wherein the plant is a flower.

31. (New) The method of claim 26, further including the step of selling the binucleate *Rhizoctonia* strain to a farmer.

32. (New) The method of claim 26, further including the step of selling the binucleate *Rhizoctonia* strain to an entity other than a commercial grower.

33. (New) The method of claim 26, wherein providing includes obtaining the sample from a public or private research institution or corporation.

34. (New) A method of protecting a seed, plant, or seedling, the method comprising providing a seed, plant, or seedling associated with a binucleate *Rhizoctonia* strain, characterized as having an ITS1 sequence which is at least 90% homologous with SEQ ID NO:20.

35. (New) A method of protecting a seed, plant, or seedling, the method comprising:
 providing a mycelium of a binucleate *Rhizoctonia* strain, characterized as having an ITS1 sequence which is at least 90% homologous with SEQ ID NO:20;
 mixing the mycelium with a diluent; and
 contacting the diluted mycelium with the seed, plant, or seedling.

REMARKS

Claims 1-6 are pending and claims 7-10 are withdrawn from consideration as being drawn to nonelected subject matter. Claims 1-10 are cancelled and claims 11-35 have been added. Thus, claims 11-35 are pending in the application.

Support for the new claims may be found throughout the specification, e.g., at page 4, line 1 through page 6, line 14 and claims 1-10 as originally filed.

Applicants have amended the Specification as indicated above to correct errors. SEQ ID NOS. 1 to SEQ ID NO. 14 are not, in fact, PCR primers, but rather characteristic sequences of the various isolates. SEQ ID NOS. 15 to 25 are ITS sequences from various isolates. When looking at the ITS sequences according to SEQ ID NOS. 15 to 25, it would be obvious to the skilled person that SEQ ID NOS. 1 to 14 were target sequences from the isolates themselves, and not primers directed thereto. The skilled person would recognise that the sequences according to SEQ ID NOS. 1 to 14 were disclosed within SEQ ID NOS. 15 to 25 and were not complimentary to sequences within these SED ID NOS.

Therefore, it would be obvious to said person that SEQ ID NOS. 1 to 14 are target sequences to which the design of complementary primers should be directed. Accordingly, we submit that the amendments to line 1, page 5 and line 15, page 14 of the international application as published are obvious corrections of an obvious error.

Claims 1-6 are rejected under 35 U.S.C. 112, second paragraph as being indefinite. According to the Office Action, "Claims 1-6 provide for the use of *Rhizoctonia*, but, since the claim does not set forth any steps involved in the method/process, it is unclear what method/process applicant is intending to encompass" (Office Action, page 2, lines 18-20).

Applicants have cancelled claims 1-6, thus rendering the rejection moot. Applicants respectfully request that the rejection not be applied to new claims 11-35. New claims 11-25 are drawn to a method of protecting plants from pathogenic fungi. The claim sets forth several steps, notably that the *Rhizoctonia* which is the active agent be provided and further that the active agent be characterized or identified as having a certain property, and allowing the *Rhizoctonia* to grow with the plant. Claims 26-33 are drawn to a method of selecting a *Rhizoctonia* strain for

the ability to protect a plant. The claim sets forth several steps, notably that a candidate *Rhizoctonia* to be analysed for protective ability is provided, nucleic acid of the candidate strain is compared with a standard, and if it meets the standard it is selected. Claim 34 is directed to a method of protecting a seed, plant, or seedling by providing it in association with a *Rhizoctonia* which has been characterized as having the recited property. Claim 35, is directed to a method of protecting a seed, plant, or seedling by providing a mycelium having the recited properties, mixing the mycelium with a diluent, and contacting with a plant, seed, or seedling. Thus, it is clear what steps must be performed to practice the method and infringe the claim.

Claims 1-6 are rejected under 35 U.S.C. 101 "because the claimed recitation of a use, without setting forth any steps in the process, results in an improper definition of a process..." (Office Action, page 2, lines 28-30)

Applicants have cancelled claims 1-6, thus rendering the rejection moot. Applicants respectfully request that the rejection not be applied to new claims 11-35. As is discussed in the context of 35 U.S.C. 112, second paragraph, the new claims include steps and define the claimed process.

Claims 5-6 are rejected under 35 U.S.C. 112, first paragraph for lack of enablement.

Applicants have cancelled claims 1-6, thus rendering the rejection moot. Applicants point out that new claims 11-35 are directed to the use and evaluation of binucleate *Rhizoctonia* strains for plant protection, which strains are characterized as having an ITS1 sequence which is at least 90% homologous with SEQ ID NO: 20. The critical aspects of the claim are the realization that having a certain level of homology with a specific sequence is useful and the ability to make the sequence comparison. The specification enables both. It explicitly discloses the relationship between protective ability and it provides the sequence to which the comparison is made. One of ordinary skill in the art can, without undue experimentation, determine the sequence of the ITS1 gene of a candidate and then determine if it has 90% homology to the disclosed standard, SEQ ID NO: 20. This sequence is taught in the specification in Figure 1, line 6 and relates to the Eab F1 isolate of the species *Ceratobasidium Albasitensis*. This taxon is part of the *Rhizoctonia* s.l. species complex and genus *Ceratobasidium*. A description of the Eab F1

strain may be found in, e.g., Gonzales et al. "*Ceratobasidium Albasitensis*: A New *Rhizoctonia*-like fungus isolated in Spain" *Persoonia* 2002, 17, 601, and a phylogenic tree, which are submitted as Exhibits A and B, respectively. Applicants respectfully request that the rejection not be applied to new claims 11-35.

Claims 1-6 are rejected under 35 U.S.C. 112, second paragraph as being indefinite. Specifically, claim 1 "is confusing in that it is unclear what is intended by 'selected by molecular detection of the ITS ribosomal sequence'" (Office Action, page 3, lines 22-23). Claim 2 is "improper in the use of punctuation in the middle of the claim" (Office Action, page 3, line 26). Claim 3 is "confusing and inconsistent in the recitation of multiple 'or' within the listing" (Office Action, page 3, lines 31-32).

Applicants have cancelled claims 1-6, thus rendering the rejection moot. Applicants respectfully request that the rejection not be applied to new claims 11-35.

Claims 1-4 are rejected under 35 U.S.C. 102(b) as being anticipated by Herr, 1988 (Herr) and Cardoso et al., 1987 (Cardoso). According to the Office Action:

...[t]he composition used in the process is claimed as a product-by-process. Since the Patent and Trademark Office is not equipped to manufacture products by the myriad of processes put before it and then obtain prior art products and make comparisons therewith, a lesser burden of proof is required to make out a case of prima facie anticipation (Office Action, page 4, lines 34-38)

Applicants have cancelled claims 1-4, thus rendering the rejection moot. As discussed above, new claims 11-35 relate to the use and evaluation of binucleate *Rhizoctonia* strains for plant protection in which the strains are characterized, identified, or selected, as having an ITS1 sequence which is at least 90% homologous with SEQ ID NO:20. This feature is neither taught nor suggested by either Herr or Cardoso. Applicants therefore respectfully request that the rejection not be applied to new claims 11-35. Nothing in the references associates protective ability with a strain which has been or is identified, selected, or characterized as having any level of homology with SEQ ID NO:20.

Claims 1-6 are rejected under 35 U.S.C. 103(a) as being unpatentable over Herr taken with Cardoso and Boysen et al. (Office Action, page 4, lines 40-41). According to the Office Action:

Herr and Cardoso et al. each discloses the protection of plants from fungi by using biocontrol with binucleate *Rhizoctonia* ... The references are silent as to whether or not the strains used were selected by molecular detection of the ITS ribosomal sequence. However, it is clear that the binucleate *Rhizoctonia* strains used in the reference are effective in the protection of plants from fungi as claimed. Moreover, Boysen et al. adequately demonstrate that the technique of the identification of the ITS ribosomal sequence is old and well known in the art for the identification and/or selection of *Rhizoctonia*. There is no clear correlation between strains having at least 90% homology to a sequence of SEQ ID NO. 20 and protective properties of biocontrol against fungi... Therefore, it would have been obvious to one having ordinary skill in the art at the time that the invention was made to modify the process of protection of Herr or Cardoso by using strains selected according to the method of Boysen et al. for the expected benefit of more predictably identifying binucleate *Rhizoctonia* suitable for biocontrol of fungi on plants (Office Action, page 5, lines 4-13 and 23-27).

Applicants have cancelled claims 1-6, thus rendering the rejection moot. However, Applicants disagree with the rejection for the following reasons.

First, according to the Gonzales reference referred to earlier (see Exhibit A), "Many [*Rhizoctonia*] isolates are also effective in protecting plants from a number of fungal diseases... Conversely, many other isolates cause significant losses in agriculture and forestry," (Gonzales, page 601, lines 7-9). Thus, the art teaches that some, but not all *Rhizoctonia* strains are *a priori* suitable for plant protection. Thus, not all strains are *a priori* useful for plant protection.

Applicants have discovered that binucleate *Rhizoctonia* strains useful for plant protections can be identified and selected on the basis of ITS data, e.g., ITS1 data. The Examiner correctly points out that the Herr and the Cardoso references state that binucleate *Rhizoctonia* strains can be useful in the protection of plants from pathogenic fungi. However, Herr and Cardoso do not teach or suggest that binucleate *Rhizoctonia* strains having a particular

ITS1 sequence or homolgy to a particular sequence would be suitable for plant protection. In fact, Herr and Cardoso are silent altogether on ITS sequences, in general.

Boysen *et al* teaches that ITS1 sequences are known in the art. However, Boysen *et al* merely states that ITS1 sequences are a useful way of identifying particular strains of *Rhizoctonia* and differentiating them from other strains. However, Boysen makes no reference to plant protection. This contrasts with Applicants' claimed methods, which use strains which have been or are characterized, identified or selected by this data as useful for plant protection.

The combination of Boysen, Herr, and Cardoso therefore only teaches one of skill in the art that certain strains of binucleate *Rhizoctonia* are useful in plant protection (which is already known in the art, see Gonzales above) and that ITS1 sequences are useful for differentiating one strain of binucleate *Rhizoctonia* from another, but not for differentiating or supplying strains on their ability to protect plants from fungal disease. There is no teaching or suggestion in any of the three references alone or in combination to identify, supply, or select binucleate *Rhizoctonia* strains useful for plant protection on the basis of ITS1 sequence data, e.g., in particular, selection of a strain having an ITS1 sequence with 90% homology to SEQ ID No. 20. Thus, Applicants submit that the Office has not established a *prima facie* case of obviousness because there is no suggestion in Herr, Cardoso, or Boysen of the desirability to modify or combine these references. Applicants respectfully request that the rejection not be applied to new claims 11-35.


Applicant : Victor Rubio Susan et al.
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Enclosed is a \$174.00 check for excess claim fees and a \$930.00 check for the Petition for Extension of Time fee. Please apply any other charges or credits to deposit account 06-1050, referencing Attorney Docket No.: 15605-002001.

Respectfully submitted,

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CERATOBASIDIUM ALBASITENSIS

A new *Rhizoctonia*-like fungus isolated in Spain

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Ceratobasidium albasitensis is described as a new species based on morphological data and on phylogenetic reconstruction from rDNA sequences, including representatives of other related species. Isolates of this species were found in several localities within Albacete province (SE of Spain). This taxon is part of the *Rhizoctonia* s.l. species complex and is placed in the genus *Ceratobasidium* (Stat. Anam. = *Ceratorhiza*) on account of its macro- and micromorphological features. In addition, two new methods to induce sexual sporulation on plates are described.

The form-genus *Rhizoctonia* is currently considered to be a heterogeneous assemblage of fungal taxa, which do not form asexual spores, but have certain significant morphological characteristics in common. The *Rhizoctonia* complex is now split into at least seven or eight genera, according to different authors (Moore, 1987; Andersen, 1996). *Rhizoctonia* s.l. has considerable ecological and economic importance because it occurs worldwide and different isolates within the complex may live as saprotrophs or as symbionts (such as those associated with terrestrial orchids). Many isolates are also effective in protecting plants from a number of fungal diseases (Sneh, 1998), promoting plant growth. Conversely, many other isolates cause significant losses in agriculture and forestry; currently *Rhizoctonia* diseases have been described in more than 200 plant species. At least 120 epithets referring to *Rhizoctonia* can be found in the literature, where until recently very few attempts to clarify genera and species concepts had been made (Sneh et al., 1991; Andersen & Stalpers, 1994; Roberts, 1999).

Teleomorphs of fungi that show the morphological characteristics of *Rhizoctonia* species with binucleate hyphal compartments have usually been reported as belonging to *Ceratobasidium* D.P. Rogers, with the exception of *Epulorhiza repens* (N. Bernard) Moore (= *Rhizoctonia repens* Bernard), which has been found by some authors to have a perfect state in *Tulasnella* Schroet. (*T. deliquescens* (Juel) Juel = *T. calospora* (Boud.) Juel s. auct.) (Sneh et al., 1991). *Ceratobasidium* is regarded as close to *Thanatephorus* Donk, being considered by some authors (e.g. Stalpers & Andersen, 1996; Roberts, 1999) as part of a generic complex, where delimitation presents some difficulties. Anamorphic *Rhizoctonia*-like fungi from these two genera have traditionally been classified on the basis of the number of nuclei per hyphal compartment, considering *Thanatephorus* as multinucleate and *Ceratobasidium* as binucleate, although the nuclear condition remains unknown for many taxa. Another important handicap to understanding the taxonomy of these fungi is the fact that most studies on *Rhizoctonia* s.l. are performed with cultures where sexual reproductive structures are

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usually not or hardly observed, so that delimitation of species is based on the morphology and physiological features of the asexual morphs. Because of this, the development of new methods for inducing teleomorph formation constitutes a valuable tool to clarifying and correlating *Rhizoctonia* taxonomy.

Some non-pathogenic or hypovirulent isolates within the form-genus *Rhizoctonia* have been shown to be highly effective biocontrol agents (Sneh, 1998). In the majority of studies to date, most of the known isolates capable of protecting plants and preventing diseases have been found to belong to *Ceratobasidium*. In preliminary studies (data not shown), isolates of the new species described here have proved to possess protective properties in several plant species against pathogenic isolates of *Rhizoctonia solani* Kühner (= *Thanatephorus cucumeris* (A.B. Frank) Donk), and other fungal pathogens such as *Fusarium* spp., *Alternaria* spp., *Penicillium* spp., etc.

As stated by some authors (e.g. Mordue et al., 1989; Andersen, 1996), the employment of integrated approaches to solve taxonomic problems in *Rhizoctonia* (including morphometrical, cultural, biochemical, ecological and molecular data) could lead to establishing more accurate and natural classifications within this group of organisms. In the present work, molecular phylogeny of binucleate *Rhizoctonia* isolates was undertaken based on sequence data from the ribosomal ITS region. The isolates included those from Albacete, several testers used for defining anastomosis groups within binucleate *Rhizoctonia*, and miscellaneous sequences of *Ceratobasidium* retrieved from GenBank. The results provided additional evidence (at sequence level) for validating the new taxon presently described.

METHODS

Ceratobasidium albasitensis isolates

Root samples of saffron corms and pine seedlings were collected from six locations in Albacete. *Ceratobasidium* isolates were collected by washing roots with tap water to remove adhering soil particles and placing both root segments and rhizobial soil particles on 1.5% water agar amended with chloramphenicol (250 mg/ml). Some of the resulting colonies were identified as belonging to *Ceratohiza* s. Moore (1987) (= binucleate *Rhizoctonia*) by observing typical morphological features such as hyphal branching, number of nuclei, colour of the cultures, sclerotia formation or dolipore septa. Cultures were then transferred and maintained on potato-dextrose-agar (PDA) at room temperature. The number of nuclei per cell was determined using a staining method as described by Julián et al. (1997).

Formation of perfect state

Two undescribed methods were used to induce formation of basidiocarps by the isolates. The first method consisted of a modification of the one described by Hietala et al. (1994). In this first procedure, sterile radish seedlings (ethanol 70% 2'; sodium hypochloride 5% with Tween 0.005% 15' and at least five washes in distilled water), were pregerminated by incubating them on 15% water-agar plates at 24°C in the dark. After two days, the seedlings were transferred to Petri-dishes containing 20 ml sterile water with two 5 × 5 mm blocks of a 7 days old colony of the fungus, and incubated at room temperature with natural lighting. Teleomorph production started between 3 and 10 days after fungal inoculation.

A second new method was developed in our laboratory, consisting of a modification of some of the previously described methods (i.e. Flentje, 1956; Murray, 1982, 1984) based

on transferring isolates from high nutrient agar to low nutrient agar. Briefly, basidiomes were obtained by growing cultures of the isolates on PDA (potato-dextrose-agar) for 5–6 days and then transferred to plates of 15 % water-agar containing small pieces (two blocks of 10 cm² aprox. per plate) of leaves or twigs from several plant species such as *Nerium oleander* L., *Prunus laurocerasus* L., *Ligustrum vulgare* L., *Pinus halepensis* Miller, etc., previously surface sterilized as described above. Hymenial production started (depending upon the strains) between 7 and 15 days after the transfer of the colonies.

Phylogenetic reconstruction

For phylogenetic analysis, the entire sequence of the ITS regions of 37 binucleate *Rhizoctonia* isolates, representing several *Ceratobasidium* teleomorphs, was determined. The sequences were aligned and processed for phylogenetic reconstruction. All the isolates used in this work are listed in Table I, including the isolates of *C. albasitensis*, all available AG testers of binucleate *Rhizoctonia* described up to date and some *C. cornigerum* and *C. cerealis* isolates from the CBS collection (Baarn, The Netherlands). In addition, several ITS sequences including *C. oryzae-sativae*, *C. cerealis*, *Waitea circinata* and *Serendipita vermifera* s. Roberts (1999) (= *Sebacina vermifera*) were retrieved from GenBank. DNA isolation, PCR and DNA sequencing procedures used have been previously described (Boysen et al., 1996). Alignments of the ITS regions were performed using the multiple alignment program CLUSTALW (Thompson et al., 1994). Phylogenetic analysis of the aligned sequences was performed using maximum-parsimony with the heuristic-search algorithm of the Phylogenetic Analysis Using Parsimony (PAUP) program 3.1.1 (Swofford, 1993) with gaps treated as missing data. The trees were rooted with *Agaricus bisporus* as an outgroup. The data were resampled with 1000 bootstrap replicates (Felsenstein, 1985) by using the heuristic search option of PAUP. The percentage of bootstrap replicates that yielded each grouping was used as a measure of statistical confidence. A grouping found in 90% of bootstrap replicates was considered statistically significant. Similar trees were also obtained from the distance matrix of Jukes and Cantor using the neighbour-joining method of the program PHYLIP 3.5 (Felsenstein, 1993).

Ceratobasidium albasitensis V. González & V. Rubio, spec. nov. — Fig. 1

Basidiocarpus albidus, resupinatus, sparsus, inconspicuus, tenuis, pelliculosus vel pulveraceum quando recens et in aridus. Hyphae subiculares hyalina, laxa, partim incrassate tunicatae, ramis angulis rectis, fibulae destitutae, 3.5–5.5(–6.5) µm latae. Basidia subglobosa vel sphaericopedunculata, producta aut singula aut quasi racemis aggregata ex hyphis subicularibus, (16.2–)18.2–20.8(–24) × 7–11 µm, 4(–5) sterigmata, longissima, cornuta, subcurvata, saepe cum septis adventitis prope apices (19–)22–35.7 × 1.9–3 µm. Sporae subglobosae vel latae ellipsoideae (Q = 1.2–1.4), laeves, hyalina, inamiloideae, raro iterativae prope laterales, 5–7(–8) × (3.5–)4–5.5(–6) µm. Hyphae moliniformiae amplae, tumoribus, 13–15 µm latae. Status anamorphosis Ceratorrhiza.

In terra ad *Crocus sativus* L. et *Pinus halepensis* Miller.

Holotypus: Hispania, Albacete, Tobarra, 7 Nov. 1996, O. Salazar & M.C. Julián, in herb. Alcalá (AH 26603) conservatur.

Etymology: referring to the geographical origin of the isolates.

Among the nine isolates identified as a new species, three of them fructified repeatedly with both of the two methods used for teleomorph induction. Thus descriptions of both macro- and microscopical characters from the sexual stage are based on fructifications from these three isolates.

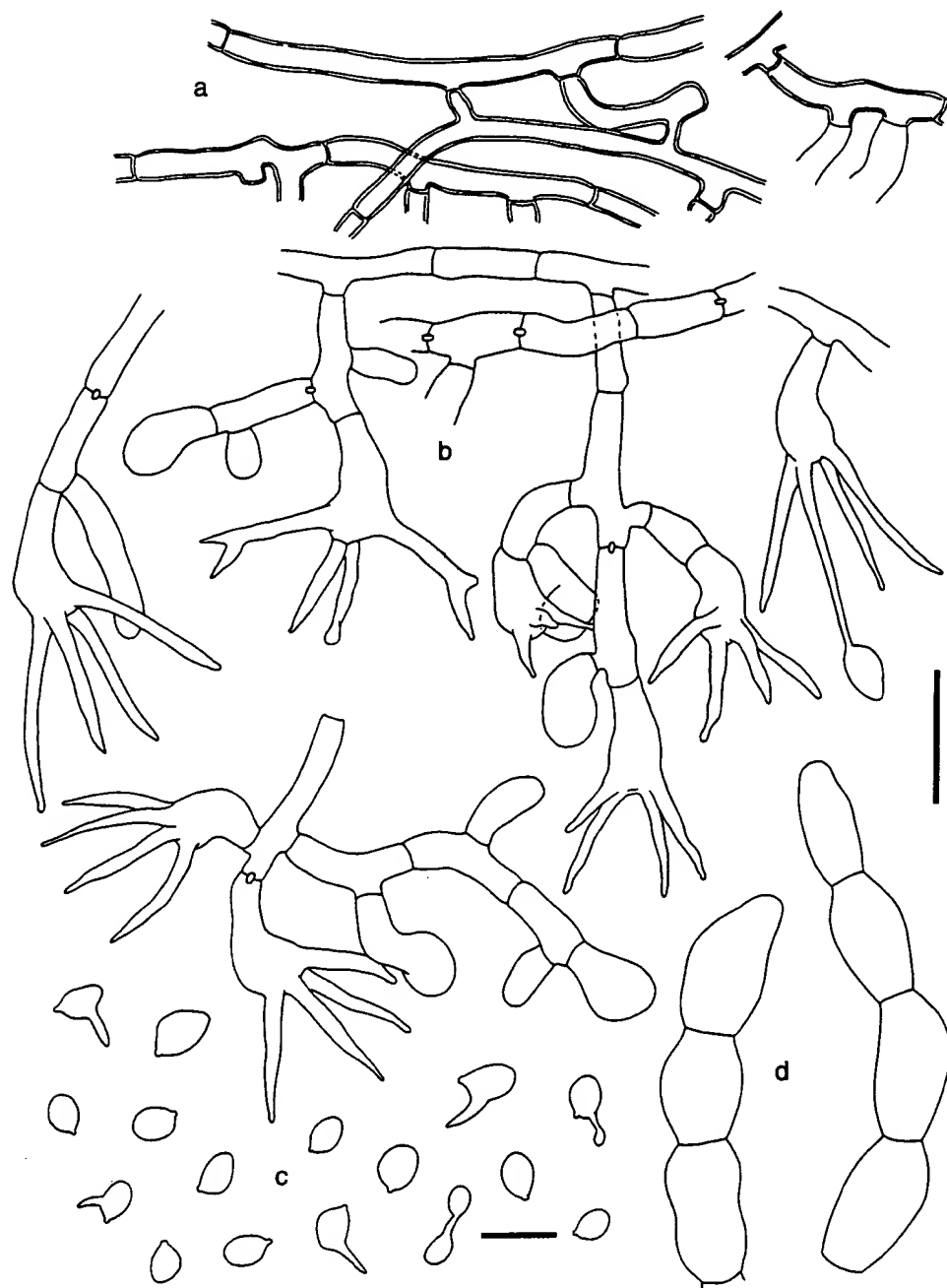


Fig. 1. *Ceratobasidium albasitensis*. a. Subicular hyphae; b. hymenial layer (basidia and basidioles); c. spores; d. molinioid cells. Bars: 10 & 20 μm respectively.

Table I. Isolates used in this study; host, size of ITS1, ITS2, total rDNA-ITS region, and EMBL or GenBank accession numbers. a) Isolates of *C. albasitensis*; b) tester isolates of binucleate *Rhizoctonia* (*Ceratobasidium* spp.); c) other isolates.

a) Isolate	Isolated from	ITS1	ITS2	Total	Accession No.	
Eab-F1	<i>Crocus sativus</i>	184	231	570	AJ242875	
Eab-F2	<i>Crocus sativus</i>	185	230	570	AJ242876	
Eab-F3	<i>Crocus sativus</i>	184	230	569	AJ242877	
Eab-F5	<i>Crocus sativus</i>	184	230	569	AJ242878	
Eab-F6	<i>Crocus sativus</i>	184	230	569	AJ242879	
Eab-F7	<i>Crocus sativus</i>	184	232	571	AJ242880	
Eab-S1	<i>Crocus sativus</i>	185	231	571	AJ242881	
Eab-S5	<i>Crocus sativus</i>	184	230	569	AJ242885	
Eab-S6	<i>Pinus halepensis</i>	184	230	569	AJ242886	
b) Isolate	AG	Isolated from	ITS1	ITS2	Total	Accession No.
C-662	AG-A	Soil	188	223	566	AJ242890
BN4	AG-A (CAG2)	Soil	188	223	566	AJ242891
SIR-2	AG-Bo	<i>Ipomoea batatas</i>	185	230	570	AJ242892
C-455	AG-Bb	<i>Oryza sativa</i>	185	230	570	AJ242893
70B	AG-C	Soil	189	223	567	AJ242894
C-610	AG-D	unknown	189	223	567	AJ242895
Rh155	AG-E	unknown	190	223	568	AJ242896
C-653	AG-G	unknown	183	232	570	AJ242897
AV-2	AG-I	<i>Artemisia sp.</i>	183	229	567	AJ242898
184	AG-J	unknown	183	229	567	AJ242899
FA59209	AG-K	unknown	193	237	585	AJ242900
76146	AG-L	unknown	190	222	567	AJ242901
TC1	AG-N	unknown	186	230	571	AJ242902
76150	AG-P	unknown	187	230	572	AJ242903
c) Isolate	Isolated from	ITS1	ITS2	Total	Accession No.	
<i>R. cerealis</i>	<i>Poa annua</i>	212	231	598	AF063019	
C. o-s 2	<i>Oryza sativa</i>	226	249	630	AJ000192	
C o-s 1	<i>Oryza sativa</i>	227	249	631	AJ000191	
C o-s 3	<i>Oryza sativa</i>	226	249	630	AJ000193	
C o-s 4	<i>Oryza sativa</i>	226	248	631	AJ000194	
Rh2815	<i>Vicia faba</i>	188	228	571	U19962	
521	soil	190	230	575	U19950	
C1 (CAG-1)	<i>Festuca</i>	198	241	594	AJ301903	
C2 (CAG-2)	<i>Pittosporum</i>	198	238	591	AJ301899	
C4 (CAG-3)	<i>Juniperus</i>	191	243	589	AJ301900	
C5 (CAG-5)	<i>Taxus</i>	190	240	585	AJ301901	
C6 (CAG-6)	<i>Erigeron canadensis</i>	185	236	576	AJ301902	
C8 (CAG-7)	<i>Pittosporum</i>	190	245	590	AJ302006	
C11	<i>Triticum aestivum</i>	210	231	596	AJ302007	
C13	<i>Triticum aestivum</i>	210	231	596	AJ302009	
C12	<i>Triticum aestivum</i>	210	231	596	AJ302008	
Eab-aB	<i>Medicago sativa</i>	228	242	625	AJ302010	
Eab-S3	<i>Crocus sativus</i>	185	233	573	AJ242883	
<i>W. circinata</i> I	<i>Oryza sativa</i>	213	198	566	AJ000196	
<i>W. circinata</i> II	<i>Oryza sativa</i>	212	198	565	AJ000195	
<i>A. bisporus</i>	unknown	294	207	656	AJ301619	
<i>S. vermifera</i>	<i>Orchidaceae</i>	171	199	525	AF202728	

Basidiomata whitish to almost hyaline, resupinate, thin, hymenium consisting of a hypochnoid, pruinose, pellicular layer over the water surface (when fruiting in sterile water with radish seedlings) or on the outer surface of the agar, near the margins of the Petri-dish, more rarely covering the whole plate surface and even occasionally colonizing vegetable debris added to the medium (i.e. pine twigs, *Nerium* leaves, etc).

Basidia globose when young, then pyriform to sphaeropedunculate $(16.2-18.2-20.8(-24) \times 7-11 \mu\text{m})$, produced directly from basal hyphae or in raceme-like groups. Sterigmata 4 or 5, very long (more than twice the length of the basidium), $(19-)22-35.7 \times 1.9-3 \mu\text{m}$, cornute, curved or straight, easily collapsing and often with adventitious septa occurring near the tips. Basidiospores broadly ellipsoid to ovoid ($Q = 1.2-1.4$, average 1.26) in front view, amygdaliform to slightly citriform in side view, $5-7(-8) \times (3.5-)4-5.5(-6) \mu\text{m}$ (average $6.42 \times 4.19 \mu\text{m}$) ($n = 21$), apiculate, hyaline, smooth, not amyloid, germinating by a lateral germ-tube and more rarely by repetition. Subicular hyphae hyaline, thick to thin-walled, $3.5-5.5(-6.5) \mu\text{m}$ in diam. Monilioid hyphae occurring among the hymenial tissues, consisting of inflated, barrel-shaped elements, up to $15 \mu\text{m}$ in diam. Clamp-connections absent in all tissues.

Habitat — Saprotrophic on rhizospherical soil and healthy saffron corms (*Crocus sativus* L.) and pine seedlings (*Pinus halepensis* Miller) in agricultural ground.

Material studied. SPAIN: Albacete, Tobarra, 28 Nov. 1996, in agricultural ground with *Crocus sativus* L., leg. O. Salazar & M. C. Julián (holotype, AH 26603); ibidem, 7 July 1996, AH 26605; Albacete, Aguas Nuevas, 7 July 1996, leg. O. Salazar & M. C. Julián, AH 26604.

A system of anastomosis grouping based on hyphal fusion is widely accepted as the basis for recognizing groups among the several taxa that constitute the form-genus *Rhizoctonia* (Ogoshi, 1975; Sneh et al. 1991). These methods have been extensively employed for both multinucleate and binucleate *Rhizoctonia* isolates, instead of taxonomical approaches dealing with teleomorph-based systems. Several authors have pointed out the difficulty in differentiating species within *Ceratobasidium* (Ogoshi et al., 1983). Although there are some reports in the literature of well-defined taxa based on the morphology of the teleomorphic phase [i.e. *C. oryzae-sativae* Gunnell & R. K. Webster (1987); *C. ramicola* Tu, Roberts & Kimbrough (1969)], many of the teleomorphs from the different anastomosis groups recognized within 'binucleate *Rhizoctonia*' (AG-E, AG-L, AG-I, AG-K, etc) obtained in the laboratory by indirect methods, although assigned to *Ceratobasidium*, are generally not defined at species level (Sneh et al., 1991).

Thanatephorus, the teleomorphic stage of multinucleate *Rhizoctonia* (e.g. *R. solani*) is the closest genus to *Ceratobasidium*. The systematic position of both genera within the Basidiomycetes has been previously studied (Talbot, 1965; Eriksson & Ryvarden, 1973; Stalpers & Andersen, 1996; etc.) and the relationships of the *Ceratobasidiaceae* with the Heterobasidiomycetes (mainly *Tulasnellaceae* and *Tremellaceae*) profusely discussed. The systematic arrangement still remains unclear, although they are presently placed among the *Ceratobasidiaceae* (Ceratobasidiales, Basidiomycetes) within the Homobasidiomycetes (Hawksworth et al., 1995). Recently, Roberts (1999) suggested a rearrangement of the classification proposed in Hawksworth et al. (1995). He proposed simplifying the current nomenclatural status of genera within the *Ceratobasidiaceae* by recognizing just three genera within the family: *Ceratobasidium*, *Thanatephorus* and *Waitea*. As stated by several authors (e.g. Stalpers & Andersen, 1996; Roberts, 1999), the differences used to distinguish these genera are gradual, although it is commonly accepted that *Thanatephorus* is applied to those

mostly parasitic fungi with hypochnoid, sometimes gelatinized basidiomes with ellipsoid to barrel-shaped basidia formed from vertically branching, cymose hyphae (mostly containing more than two nuclei per compartment), while the name *Ceratobasidium* is applied to taxa with ceraceous fruitbodies with ovoid to sphaeropedunculate basidia arising in raceme-like groups from the subicular hyphae (generally with two nuclei per compartment). The convenience of accepting genus *Waitea* as a member of the *Ceratobasidiaceae* will be discussed below.

The sizes of the ITS region (ITS1, 5.8S and ITS2) of the different isolates are listed in Table 1. The ITS1 region varied in length from 171 to 228 bp, the ITS2 region varied from 198 to 249 bp. It was regularly observed that the ITS1 region was slightly shorter than the ITS2 for all the isolates used in the study. The 5.8S region was highly conserved: all the isolates analyzed were 155 bp in length, only minor variations in nucleotide sequence were detected among isolates and this variation was identical in isolates belonging to the same species. The percentage of similarity in the total ITS region among the main clusters recognized in the phylogenetic tree (labelled from G1 to G9) is summarized in Table II.

Phylogenetic trees based on ITS sequences were obtained with PAUP. PHYLIP analysis did not differ in the topology of the strongly supported branches. The parsimony analysis of these characters generated 100 equally parsimonious trees with the minimum length tree of 1008 steps, with consistency (CI) and retention (RI) indexes of 0.6577 and 0.8214 respectively. One of these most parsimonious trees is shown in Fig. 2. Parsimony analysis of the ITS region supports in general the morpho- and cytological criteria used for defining and delimitating species within *Ceratobasidium*.

With the exception of *Ceratobasidium cornigerum* (AG-Bo and AG-P), *C. cerealis* (AG-D) and *C. oryzae-sativae* (AG-Bb), when more than one isolate of the same species was sequenced, they always clustered together in the same branch, or at least in adjacent branches.

Phylogenetic analysis grouped all the binucleate taxa with a *Ceratobasidium* teleomorph in seven groups, most of them well supported by bootstrap indexes. These binucleate groups clustered apart from the isolates of *Waitea circinata* and *Serendipita vermifera*. Several authors have discussed the position of these last two taxa, usually considered within the concept of *Rhizoctonia* s.l. according to the morphological features exhibited by their anamorphic states. Moore (1978, 1987), employing electron microscopy to characterize the ultrastructure of the septal pore apparatus, designated those binucleate *Rhizoctonia* anamorphs with a perfect state in the genus *Tulasnella* as belonging to *Epulorhiza*, while Andersen and Moore (Moore, 1996) erected the ephitet *Opadorhiza* to accommodate *Rhizoctonia globularis* Saksena & Vaartaja, a taxon with a teleomorph in *Sebacina* s.l., very similar to *Epulorhiza* but with a different septal pore apparatus.

On the basis of these studies, both genera must be considered as peripheral to *Rhizoctonia* in the modern concept of the complex. Nevertheless, Roberts (1999) included *Waitea* as a member of the *Ceratobasidiaceae*, considering the genus as very close to (if not synonymous with) *Thanatephorus* based on morphological criteria. Phylogenetic reconstruction carried out in the present study is not in concordance with this hypothesis, and suggests moving *Waitea* out of the *Ceratobasidiaceae*. Furthermore Johanson et al. (1998), in a study employing a PCR-based method to discriminate between the different taxa involved in the rice sheath-blight disease complex, showed that isolates from *Rhizoctonia oryzae* (teleomorph = *Waitea circinata*) clustered apart from those of *R. solani* and *R. oryzae-sativae*.

Tree length = 1008
 CI = 0.6577
 RI = 0.8214
 RC = 0.5403

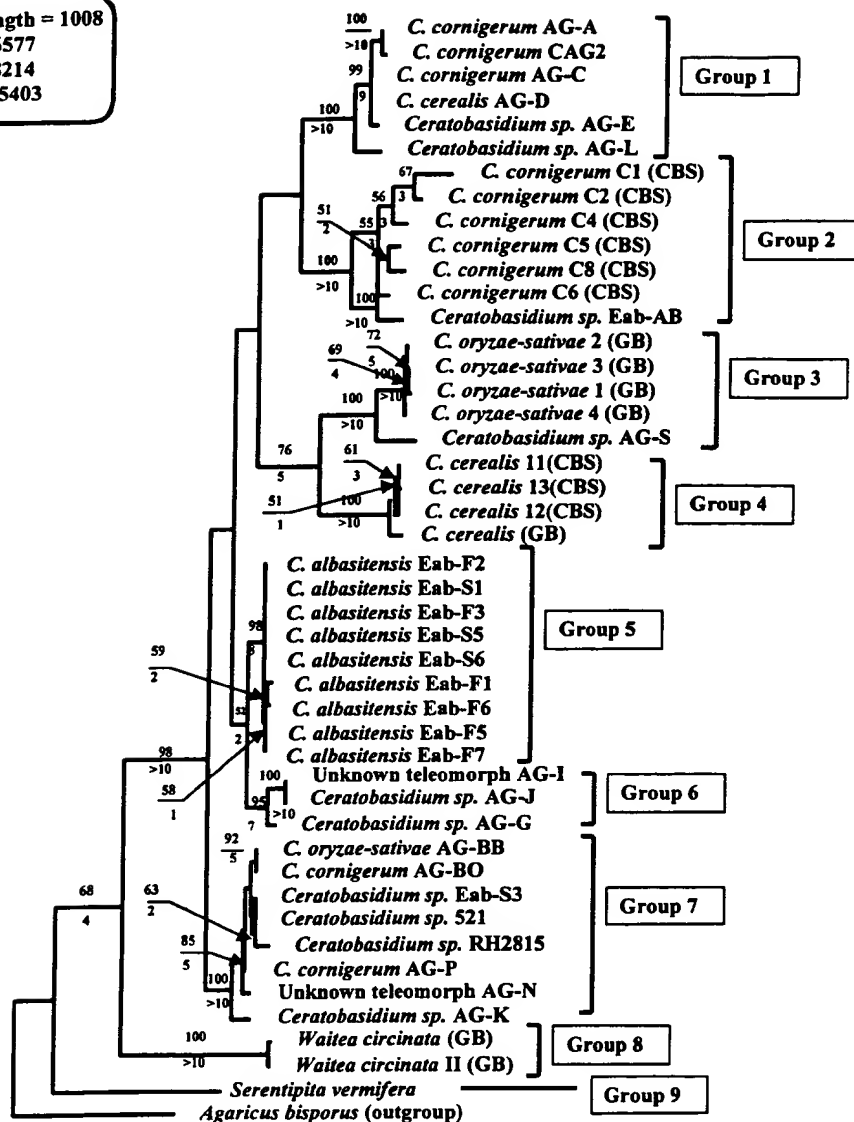


Fig. 2. One of the most parsimonious trees generated from a branch and bound algorithm in PAUP 3.1.1, using CLUSTALW for alignment. The trees were rooted with the sequence of one isolate of *Agaricus bisporus* (Agaricaceae). The numbers above branches indicate the bootstrap percentage from 1000 bootstrap replicates (for values greater than 50%). Decay indices up to 10 steps are indicated below branches. Horizontal lengths represent genetic distances.

Table II. Percentage of sequence divergence among the nine clusters recognized in the phylogenetic tree.

	G1	G2	G3	G4	G5	G6	G7	G8	G9
G1	0.00– 6.27	14.09– 17.84	13.03– 18.24	14.89– 17.38	10.84– 12.80	10.44– 13.02	10.48– 14.61	31.67– 33.48	40.64– 43.48
G2		3.15– 13.88	15.16– 22.80	15.75– 25.22	10.57– 14.38	12.07– 13.99	11.63– 15.94	34.07– 40.23	42.90– 44.56
G3			0.16– 8.46	16.46– 17.75	13.23– 15.85	11.80– 15.06	12.89– 17.82	36.19– 39.46	42.03– 43.61
G4				0.34– 1.53	13.84– 15.09	13.67– 15.43	14.78– 16.65	38.25– 39.19	43.01– 43.72
G5					0.00– 1.06	4.56– 6.30	6.27– 8.35	30.56– 31.52	40.32– 41.71
G6						0.18– 2.88	7.34– 10.02	29.83– 31.15	40.08– 40.32
G7							0.35– 4.37	29.61– 30.85	39.05– 41.83
G8								0.53	45.24– 45.37
G9									

(teleomorph = *Ceratobasidium oryzae-sativae*). The systematic position of *Serendipita vermifera*, as revealed by molecular phylogeny, agrees with the conclusions previously reported by some authors (e.g. Roberts, 1999; Andersen, 1996; etc). The first author segregated some taxa from the genus *Sebacina* Tul. (*Tremellales* s. Hawksworth et al., 1995) into at least four genera: *Ceratosebacina* P. Roberts, *Endoperplexa* P. Roberts, *Serendipita* P. Roberts and *Hauerslevia* P. Roberts, all of them included by the author in the Exidiales. In addition, Andersen (1996) employed both ultrastructural and molecular methods (including RFLP analysis) to distinguish *Waitea* and *Sebacina* s.l. from other forms within *Rhizoctonia*.

Group 7 includes several AG testers of binucleate *Rhizoctonia* such as AG-Bo, AG-Bb, AG-P, AG-N and AG-K, one binucleate isolate from Spain previously identified as *Ceratobasidium* sp., together with the isolates 521 and 2815, and two *Rhizoctonia* isolates previously reported in the literature as *R. solani* AG-4 (Boysen et al. 1996). This cluster separates from the rest of the binucleate isolates (100% bootstrap value), forming a heterogeneous assemblage of taxa, including two strains of *C. cornigerum*, one of *C. oryzae-sativae*, and several AG tester isolates with insufficient or completely unknown sexual morphs. In spite of the nomenclatural heterogeneity, nucleotide divergence within this branch ranges from 0.35 to 4.37%, suggesting that the taxonomic status of some of these AG testers must be revised.

Group 6 includes three AG tester isolates (AG-I, AG-G and AG-J), all of them without any well-defined teleomorph. Recently, Masuhara et al. (1994) reported the teleomorphic features of some isolates of AG-I, and characterized them as probably belonging to the species complex *Ceratobasidium cornigerum*, although these authors reported two sexual morphs, *C. cornigerum* and *C. pseudocornigerum*, for several isolates. The other two strains, AG-G and AG-J are reported in the literature (Sneh et al., 1991) as belonging to *Ceratobasidium*, without defining any specific epithet.

Group 5 brings together all the strains determined as belonging to the new species proposed here (with a 98% bootstrap value). Isolates from this branch ranged between 0.00 and 1.06% in nucleotide divergence (the lowest values found in any of the 9 clades), suggesting that strains from clade 5, although isolated from different localities (and even from different hosts) could represent a well-defined and homogeneous taxon within *Ceratobasidium*.

Groups 4 and 3 branch together (supported by a 76% bootstrap value) and contain several *Ceratobasidium cerealis* isolates (Group 4) and a set of *C. oryzae-sativae* strains (Group 3) with one binucleate AG tester (AG-S) referred to in the literature as *Ceratobasidium* sp. Group 4 included sequences obtained from CBS isolates, together with one sequence obtained from the GenBank, and all of them showed a nucleotide divergence ranging from 0.34 to 1.53%, which suggests a very low intraspecific variation for this taxon. In addition, the other isolate (AG-D) with a sexual morph defined as *C. cerealis*, clustered in Group 1, together with several *C. cornigerum* AG testers. *Ceratobasidium gramineum* (= *C. cerealis*; *Corticium gramineum*) was proposed (Oniki et al., 1986) as the teleomorphic state for AG-D (CAG-1) strains. These authors obtained some sexual fructifications from AG-D strains and compared morphological measures of their hymenial components with those of *Corticium gramineum* Ikata & T. Matsuura and *Ceratobasidium cereale* Murray & Burpee, concluding that these three taxa must be considered conspecific. After critical revision of the dimensions reported for *Corticium gramineum* and *C. cerealis* in Oniki et al. (1986), these last two taxa could not be considered as synonyms. Thus, teleomorphs exhibited by AG-D strains can be assigned to the taxa described by Murray & Burpee (1984), but not to *Corticium gramineum*. On the other hand, dimensions reported for *C. cereale* by these last authors fit into the range of measures given by Rogers (1935) for *C. cornigerum*. Furthermore, a recent study (Toda et al., 1999) on several AG-D isolates from turfgrass, differentiate two subgroups (I and II) within the AG by means of rDNA and RAPD analyses. In summary, molecular and morphological data suggest the existence of two teleomorphic forms for this AG; one of them probably closely related to other AG groups (i.e. AG-C, AG-A, etc) with a *C. cornigerum* teleomorph, and other groups defined by possessing a *C. cerealis* teleomorph.

Group 3 includes four sequences of *C. oryzae-sativae* retrieved from GenBank and one AG-S tester isolate. The original host of this last strain is not mentioned in the literature and, although no teleomorphic state has been defined up to date for this tester, it could also represent a member of this taxon. Phylogenetic reconstruction suggests that this well-known rice pathogen could constitute a natural taxon, in spite of the nucleotide divergence observed for the group (ranging from 0.16 to 8.46%) and the heterogeneous geographical origin of the samples analyzed.

Group 2 includes some strains from the CBS culture collection (with a 100% bootstrap value), all of them deposited under the name of *Ceratobasidium cornigerum*, plus one isolate from Albacete (Spain), previously identified (using teleomorph induction methods) as

C. cornigerum s.l. Within this clade, nucleotide divergence ranges from 3.15 to 13.88%, indicating high rates of heterogeneity at sequence level. Furthermore, the CBS strains were deposited as representative isolates from several of the anastomosis groups defined for binucleate *Rhizoctonia* in America (Burpee et al., 1980a; 1980b). These molecular data suggest that American AGs represent a closely related pool of taxa where the different groups could constitute populations of the same collective taxon with different rates of somatic isolation.

Group 1 and Group 2 formed a common branch. Group 1 isolates are 5 Japanese AG testers (AG-A, AG-C, AG-D, AG-E and AG-L) and one isolate from CAG2. With the exception of AG-D (discussed above), teleomorphs indicated in the literature from members of this group are mostly *Ceratobasidium cornigerum* or unnamed *Ceratobasidium* spp. Several authors (e.g. Ogoshi et al., 1983) have proposed correlating most of the American binucleate AGs with the Japanese groups. The phylogenetic reconstruction carried out in this work suggests considering the different AGs described for binucleate *Rhizoctonia* (both American and Japanese isolates) as members of the same collective taxon, where relationships between the different groups of isolates (in terms of somatic compatibility) need to be revisited, due to the fact that correlations based on hyphal anastomosis are not well reflected at sequence level. Cubeta et al. (1991), employing molecular methodologies (RFLP analysis) to characterize most of the AGs of binucleate *Rhizoctonia* species, were able to separate 13 of the 21 AGs defined for binucleate *Rhizoctonia*, although the relatedness and correlations between the American and Japanese groups were not exactly consistent with those proposed by Ogoshi et al. (1983).

With respect to the nutritional behaviour of the taxa included in the study, molecular data supports the differentiation of the several life styles within *Ceratobasidium*. Phylogenetic reconstruction suggests the existence of at least two monophyletic trends in the genus. One of them is linked to pathogenicity, and includes Group 1 and Group 2, where most of the isolates are reported in the literature as pathogenic to several plant species (Burpee et al., 1980b; Ogoshi et al., 1983; Toda et al., 1999; etc.), plus Groups 3 and 4, which include rice and grass pathogens. The other branches in the phylogenetic tree involve mostly saprotrophic taxa (Group 5), as well as other isolates not previously reported as pathogens (e.g. AG-I, AG-P, AG-N, etc.), or even described as plant protective isolates (e.g. AG-G) (Leclerc-Potvin et al., 1999).

In summary, phylogenetic analysis of the ITS region supported the definition of *Ceratobasidium albasitensis*, an undescribed binucleate taxon well differentiated from other taxa described in the genus by morphometrical methods (see discussion below). Furthermore, phylogenetic reconstruction allowed the outlining of some hypotheses on the relationships among the rest of the taxa within *Ceratobasidium*. Thus, *C. cornigerum*, the most common and widespread taxon of the genus, has been revealed in our studies as a large complex of taxa (including different species, varieties, ecotypes and populations), where a critical revision of the species concept, as well as a redefinition of the criteria for defining anastomosis groups is still required.

On account of the presence of two nuclei per hyphal compartment, saprotrophic behaviour, presence of a *Ceratorhiza* anamorph and the micromorphological features exhibited (including hymenial structure and basidial shape), we place the new species described in the genus *Ceratobasidium*. *Ceratobasidium albasitensis* is characterized by its soil habitat, large and sphaeropedunculate basidia, ovoid to subellipsoid spores and extremely large sterigmata (up to 35.7 µm), and clearly differs from other described *Ceratobasidium* species.

Table III. A microscopic comparison between *Ceratobasidium albasitensis*, *C. stridii*, *Thanatephorus obscurum* and *C. cornigerum*.

species	spores	Q	sterigmata	habitat
<i>C. albasitensis</i>	5–7(–8) × 4–5.5(–6)	1.2–4.0	–35.7 µm long	saprotrophic ¹
<i>C. stridii</i>	5.5–7.5 × 3–5	1.5–1.9	–8 µm	saprotrophic ²
<i>T. obscurum</i>	8–10 × 6–7	1.2–1.4	–1.2–1.4 µm	saprotrophic ³
<i>C. cornigerum</i>	(6.6–)7–11.5 × 3.5–6	1.9–2.0	–12 µm long	saprotrophic, parasitic and symbiotic ^{3, 4}

1) This work; 2) Eriksson & Ryvarden, 1973; 3) Roberts, 1999; 4) Rogers, 1935.

Ceratobasidium stridii J. Erikss. & Ryvarden, a rare north temperate taxon characterized by having small basidiospores of 5.5–7 × 3–4 µm, resembles *C. albasitensis*, but, based on the literature available (Eriksson & Ryvarden, 1973; Roberts, 1999), spores of *C. stridii* are oblong to fusiform (Q = 1.5–1.9). In addition, spores from *C. stridii* were reported to produce secondary spores mostly apically, a feature not constantly present in the genus and absent in the taxon presently described. The most common and ubiquitous taxon of the genus, *C. cornigerum* (Bourd.) D.P. Rogers, seems to be a complex of several different taxa, which some authors (e.g. Roberts, 1999) consider within a broad species concept. Nevertheless, the last author has pointed out a possible splitting of the complex among several of the existing synonyms on the basis of genetic distinctions. In this sense, DNA sequencing in the present study suggests separating taxa such as *C. cereale* from *C. cornigerum* s. str. Concerning the new species proposed here, *C. cornigerum* differs from *C. albasitensis* in having subceraceous basidiomes with smaller, laterally stalked cuboid to papillate basidia and larger, ellipsoid to fusiform, sometimes subcylindrical (Q = 1.4–2.0) spores up to 12.5 µm long and shorter sterigmata up to 12 µm long (Rogers, 1935; Eriksson & Ryvarden, 1973; Jülich, 1989; Roberts, 1999). *Ceratobasidium obscurum* D.P. Rogers, a taxon misreported as an orchid associate (Warcup & Talbot, 1967) and presently combined as *Thanatephorus obscurum* (D.P. Rogers) P. Roberts (Roberts, 1998), has spores resembling those of *C. albasitensis*, being subglobose to broadly ellipsoid with Q = 1.2–1.4 (but not overlapping in size range). This taxon must be definitively considered as a *Thanatephorus* species as revealed by Roberts (1998) in a study of the type collection, where this author reports oblong basidia forming hymenial palisades and a saprotrophic habitat on rotten wood of *Ulmus* sp.

Morphological measures of *C. albasitensis* and some other *Ceratobasidium* species are summarized in Table III.

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Tree length 2201

CI = 0.473

RI = 0.839

RC = 0.396

Gen. *Thanatephorus*
(*Rhizoctonia* s.str.)

Gen. *Ceratobasidium*
(binucleate *Rhizoctonia*)

Gen. *Waitea*

